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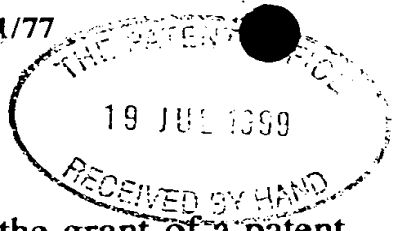

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SynGenix Limited
Home Farm
Fowlmere Road
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Herts SG8 8PG
United Kingdom

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

GB

4. Title of the invention

DEFECTIVE PACKAGING NON-ONCOVIRAL VECTORS

5. Name of your agent *(if you have one)*

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
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EC2M 7LH

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Gill Jennings & Every

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DEFECTIVE PACKAGING NON-ONCOVIRAL VECTORS

Field of the Invention

This invention relates to vectors and their use in gene transfer. The vectors are based on retroviruses, adapted so that they cannot package their own RNA, and which can be used as infectious agents to transfer foreign genes, e.g. for somatic gene therapy.

Background of the Invention

Retroviruses are classified in several ways. They are divided into various groups on the basis of their morphology. These groups are A,B,C and D type viruses. They are also classified as belonging to one of three subfamilies, namely oncoviruses, spumaviruses and lentiviruses.

SIV belongs to the family of retroviruses designated C-type viruses. These are characterised by capsid assembly at the cell membrane, and include viruses of the lentivirus group, e.g. Human Immunodeficiency Virus (HIV).

Retroviruses are RNA viruses which replicate through a DNA proviral intermediate which is integrated in the genome of the infected host cell. The virion particle contains a dimer of positive-strand genomic RNA molecules. This genomic RNA is the full-length species transcribed from the proviral DNA by the host RNA polymerase II. A proportion of these full-length RNAs which encode the *gag* and *pol* genes of the virus is translated by the host cell ribosomes, to produce the structural and enzymic proteins required for production of virion particles. The provirus also gives rise to a variety of smaller singly and multiply-spliced mRNAs coding for the envelope proteins and, in the case of more complex retroviruses, a group of regulatory proteins. The genomic (and subgenomic) RNA molecules are structurally similar to cellular mRNAs in having a 5' m^7G cap and a polyadenylated 3' tail.

A series of problems must be addressed for successful packaging of genomic RNA: The full-length RNA must be packaged preferentially over the spliced viral messages as it is the only one carrying the full complement of genetic information for the next generation of virions. The virus must also specifically select the genomic RNA against the enormous quantity and variety of physically similar host cell mRNAs as, unlike many other viruses, retroviruses do not generally arrest host RNA synthesis. There must be a mechanism whereby genomic RNA to be packaged is recognised such that a proportion is either protected from being translated and transported to an assembly site or is associated with the *gag* precursor polyprotein which it has encoded

immediately after translation. Lastly, there is the stoichiometric problem of having to package the correct number of genomes in association with 3-4000 *gag* precursor proteins, adequate numbers of reverse transcriptase molecules, a protease, tRNA primers and, in some cases, multiple copies of regulatory proteins.

Packaging the genome thus entails problems of specificity of selection of RNA and also considerations of RNA compartmentalisation.

The virus overcomes these problems by the presence of *cis*-acting elements, i.e. "packaging signals", in the viral genomic mRNA. Studies on spontaneously arising and laboratory constructed viral mutants have confirmed that specific sequences are critical for RNA recognition and encapsidation. Linial *et al*, Cell 15:1371-1381 (1978); Mann *et al*, Cell 33:153-159 (1983); Watanabe *et al*, PNAS USA 79:5986-5990 (1979) and WO-A-9119798 disclose that deletions in the 5' untranslated leader sequence lead to defects in packaging in, respectively, Rous Sarcoma Virus (RSV), Moloney Murine Leukemia Virus (MoMLV), Spleen Necrosis Virus (SNV) and HIV.

Deletion mutants have defined sequences necessary for RNA packaging in several retroviruses. In some of these, the extent of the sequence sufficient for packaging has also been mapped. Implicit in the description of packaging signals and RNA secondary structure is the premise that, if this sequence is introduced into heterologous RNA then, theoretically, the heterologous RNA should be encapsidated by retroviral particles. Constraints on packaging include the theoretical one (for which Mann *et al*, J. Virol. 54:401-407 (1985), provide some circumstantial evidence) that sequences adjacent to the packaging signal (PSI) should not favour the formation of alternative secondary structures disrupting PSI. Additionally, the total length of RNA packaged is physically limited by the capacity of the virus to package RNA of a certain size. In HIV, proviral constructs incorporating heterologous genes have been shown by Terwilliger *et al*, PNAS USA 86:3857-3861 (1989), to lead to a replication defect when the total length of the viral RNA produced significantly exceeds that of the original virus. The replication defect is consistent with a declining efficiency of RNA packaging.

Nevertheless, there is significant variability between different viruses in the nature and site of their encapsidation sequences. The mechanism of RNA recognition is so poorly understood that theoretically it is not possible to make predictions of the exact site and nature of encapsidation sequences without experimental data.

The development of retroviral vector systems has been a direct development of the work described above. In these systems, a packaging-defective "helper" virus is used to generate particles which encapsidate a highly modified RNA genome (the vector). Watanabe *et al*, Mol. Cell Biol. 3:2241-2249 (1983), and Eglitis *et al*, BioTechniques 6:608-614 (1988), report that vectors containing a minimum of the viral long terminal repeats, the packaging signal and a primer-binding site together with a heterologous marker gene have been encapsidated into virion particles and transferred to the cells for which the parent virus is tropic. By this means, it has been possible to define the minimal sequence required for encapsidation of RNA into a virus particle.

Adam *et al*, J. Virol. 62:3802-3806 (1988), disclose that, for MoMLV, the sequence sufficient for packaging encompasses the 5' leader region first defined by deletion as leading to a packaging defect. No additional sequences were essential although *gag* sequences enhanced packaging of the vector.

WO-A-9119798 discloses that an HIV-based vector containing essentially only the 5' leader sequence as a potential packaging signal was reported to be successfully encapsidated by an HIV-based packaging system. This work has not yet been confirmed. Indeed, there has been failure to encapsidate HIV RNA containing only the 5' leader sequence.

Although prior suggestions (see Rizvi and Panganiban) have been made for the approximate position of the packaging signal in SIV, by the methodology of using other viruses to package the SIV RNA, this work is fundamentally flawed for several reasons. Firstly, a considerable stretch of the SIV genome was included and, thus, there was no attempt to define the sequences sufficient for packaging. From this work, there would only be the most incomplete suggestion as to how to use SIV sequences to package heterologous genes. In addition, the region used contained many other important *cis*-acting sequences for SIV including the primer-binding site, splice donor and the complete 5' leader region. Deletion of this whole region from SIV in order to generate a packaging-defective virus would be completely unsuccessful because of the importance of other segments of this region for viral transcription, reverse transcription, splicing, etc. Thus, the work disclosed by Rizvi and Panganiban does not define sequences that are necessary or sufficient for SIV packaging to an extent which might be useful for development of SIV as a vector. Secondly, the concept of using packaging by a second virus for determining the specific packaging

sequences has recently shown to be flawed. Kaye *et al* (1998) demonstrate, for example, that HIV-1 and HIV-2 show a non-reciprocal packaging relationship such that packaging of one genome by another virus does not imply that those sequences are the relevant ones for packaging of the viral genome by its own proteins. Neither does absence of packaging of one virus by another mean that sequences included in the RNA have no relevance for packaging when this occurs in the context of the virus's own proteins.

Summary of the Invention

According to the present invention, a provirus is capable of producing SIV proteins but is not replication-competent because the RNA cannot be packaged into virions. Using this packaging-defective provirus vector, packaging-defective cell lines can be created and used to investigate the packaging mechanism of the virus and to develop strategies to interfere with this packaging mechanism. Virions produced by such packaging-negative proviruses may be used for vaccines and as a system for efficiently introducing a desired gene into a mammalian cell.

Description of the Invention

SIV has several potential advantages as a retroviral vector, compared to other retroviruses which have already been used and are being developed as gene vectors for potential use in humans. In particular:

1. It is a virus of the lentivirus family and, therefore, has the ability to enter and efficiently integrate the genes it is carrying into a cell which is not undergoing mitosis. Thus, unlike vectors based on murine or avian retroviruses, a vector system based on SIV will be able to target important tissues such as neurons, cardiac muscle and liver cells which divide rarely or never in the adult human.
2. There is no evidence that SIV is pathogenic in humans, unlike the human immunodeficiency viruses type 1 and type 2 which are being developed as lentivirus vectors for gene transfer in humans. Using HIV-1 or HIV-2 as vectors involves the chance, however remote, that during manufacture or passage or possibly in an infected individual, the vector sequences may recombine with sufficient other viral sequences to recreate a full-length infectious and pathogenic human immunodeficiency virus.
3. SIV has a genome of greater than 8 kilobases which appears to be largely redundant for packaging function and which could, thus, be replaced by the desired genetic sequences. Most cDNA copies of RNAs coding for genes

which are candidates for gene therapy would fit into a viral genome of these dimensions.

4. The molecular biology and virus assembly functions of SIV are well understood.

5. SIV can be pseudotyped with alternative viral envelopes. Thus, it has the ability to deliver genes to cells bearing the CD4 antigen using the SIV envelope itself. Alternatively, new envelopes can be adjoined to the virus in order to direct its tropism to any desired cell shape.

6. There is no apparent detectable homology within endogenous human retroviral sequences, thus minimising the potential for recombination between therapeutic vectors and endogenous sequences that would lead to replication-competent virus arising *in vitro*.

7. There is no evidence that SIV encapsidates heterologous viral RN sequences such as the VL30 family encapsidated by murine retroviral vectors and transmitted to all cells transduced by murine retroviral vector systems.

The present invention is based on studies of the molecular biology and replication of wild-type and mutant SIV and defective virus constructs made therefrom (vectors), in which segments of the 5' prime leader region have been deleted in order to localise the cis acting signals involved in viral RNA packaging in SIV. Signals both necessary and sufficient for packaging of RNA into a virion particle have been defined. It appears that the localisation of signals necessary and sufficient for RNA packaging differs from other viruses previously studied, and that the site of these signals could not have been predicted by direct analogy to other retroviruses and study of their packaging mechanism. Vectors have been constructed which themselves are replication-defective but whose RNA can be packaged in *trans* by wild-type virus and delivered to target cells for which SIV itself is tropic by infection. These vectors integrate into the target cell chromosome and express the target gene efficiently enough in the case of antibiotic resistance markers to produce target cells which themselves are antibiotic-resistant.

In particular, it has been discovered that it is possible to make SIV packaging-defective vectors. It has been found that the region between the primer-binding site and the 5' major splice donor in SIV contains sequences necessary for efficient packaging of SIV RNA into virions. In addition, it has been found that the region between the 5' major splice donor and the *gag* initiation codon contains a second and

less important region, important but not essential for packaging of SIV RNA into virions. One can prepare a vector comprising a packaging-defective SIV provirus, wherein the vector contains a nucleotide sequence which corresponds to a sufficient number of nucleotides from an SIV genome to express desired SIV products, but does not correspond to a sufficient number of nucleotides corresponding to the region between the primer-binding site and the 5' major splice donor or between the splice donor and the *gag* initiation codon to efficiently package SIV RNA (the packaging sequence).

These sequences preferably correspond to the genome of SIV. The term corresponds means that conservative additions, deletions and substitutions are permitted. The primer-binding site (23 bp) and the 5' major splice donor are respectively numbered 121-143 and 295-296 in the genomic nucleotide sequence where the transcript start site is defined as 1.

Preferably, the vector does not contain the SIV packaging sequences corresponding to the segments immediately downstream of the primer-binding site and just upstream of the 5' major splice donor and/or those immediately downstream of splice donor and immediately upstream of the *gag* gene. Typically, the vector may contain nucleotides ranging from about 20 bases of the primer-binding site to about 80 bases downstream of the primer-binding site and still be packaging-deficient and/or about 20 bases downstream of major splice donor to 70 bases downstream. In one embodiment, the packaging sequence absent from the vector contains the 85-base nucleotide sequence shown herein as SEQ ID No. 1, i.e.

GAAATAGCTGTCTTGTTACCAGGAAGGGATAATAAGATAGATTGGGAGAT

In another embodiment, the packaging sequence comprises the 50-base segment sequence shown as SEQ ID No. 2, i.e.

AGAACTCCTGAGTACGGCCTGAGTGAAGGCAGTAAGGGCGGCAGGAACCAAC
CACGACGGAGTGCTCCTATAAAGGCGCAGGTCG

The number of bases that need to be left out of the vector can vary greatly. For example, the given 50 or 80-base pair deletions in SIV are sufficient to result in loss of packaging ability. However, even smaller deletions in this region should also

result in loss of packaging efficiency. Indeed, it is expected that a deletion as small as about 5 base pairs in this region should remove efficient packaging ability. The size of a particular deletion can readily be determined based on the present disclosure by the person of ordinary skill in the art.

The vector should contain an SIV nucleotide segment containing a sufficient number of nucleotides corresponding to nucleotides of the SIV genome to express functional SIV gene products, but as described above, should not contain a sufficient number of nucleotides corresponding to the region between the primer-binding site and the 5' major splice donor or between 5' major splice donor and *gag* gene to permit efficient packaging of the viral RNA into virions. In using these vectors to establish SIV packaging-defective cell lines, it is preferred that such cell lines do not produce any infectious SIV. Although a cell line transformed by these packaging-defective deficient vectors would have low infectivity because the cells are packaging-defective, some RNA can still be packaged into the virion. Accordingly, it is preferable that the SIV nucleotide segment does not correspond to the entire SIV genome so that, if some of the viral RNA is packaged into the virion, what is packaged will not be replication-competent virus.

Preferably, a selected cell line is transformed using at least two different vectors, each containing a different portion of the SIV genome and also not containing the sequence necessary for viral packaging. Then, by cotransfecting a cell with each vector, the cell would still be able to express all the SIV structural and enzymatic proteins and produce virions. In one preferred embodiment, the or each vector does not contain sequences corresponding to an SIV LTR (long terminal repeat sequence) but contains sequences corresponding to a promoter region and/or another genome's polyadenylation sequences. Selection of particular promoters and polyadenylation sequences can readily be determined based upon the particular host cell. Preferably the LTR to which the sequences do not correspond is the 3'LTR.

In one preferred embodiment, one vector includes sequences permitting expression of SIV proteins upstream of *env* and the second vector permits expression of the remaining proteins. For example, one vector contains an SIV nucleotide segment corresponding to a sufficient number of nucleotides upstream of the *gag* initiation codon to the *env* gene sequence to express the 5'-most gene products. The other vector contains an SIV nucleotide segment corresponding to a sufficient number of nucleotides downstream of the *gag* gene sequence and including a functional *env*

gene sequence. Such vectors can be chemically synthesised from the reported gene sequence of the SIV genome or derived from the many available SIV proviruses, by taking advantage of the known restriction endonuclease sites in these viruses by the skilled artisan based on the present disclosure.

Preferably, a different marker gene is added to each vector. Then, using a preselected cell line cotransfected with these different vectors, and by looking for a cell containing both markers, a cell that has been cotransfected with both vectors is found. Such a cell would be able to produce all of the SIV proteins. Although virions would be produced, the RNA corresponding to the entire viral sequences would not be packaged in these virions. One can use more than two vectors, if desired, e.g. a *gag/pol* vector, a protease vector and an *env* vector.

Retroviruses can in some cases be pseudotyped with the envelope glycoproteins of other viruses. Consequently, one can prepare a vector containing a sufficient number of nucleotides to correspond to an *env* gene from a different retrovirus. Preferably, the 5'LTR of this vector would be of the same genome as the *env* gene. Such a vector could be used instead of an SIV *env* packaging-defective vector, to create virions. By such a change, the resultant vector systems could be used in a wider host range or could be restricted to a smaller host range, e.g. using an HIV *env* gene vector which would restrict the cell range to those bearing the CD4 protein. Using a vesicular stomatitis virus or rabies virus envelope protein would make the vector tropic for many different cell types.

Virtually any cell line can be used. Preferably, a mammalian cell line is used, for example CV-l,Hela, Raji, SW480 or CHO.

In order to increase production of the viral cellular products, one could use a promoter other than the 5' LTR, e.g. by replacing the 5' LTR with a promoter that will preferentially express genes in CV-1 or HeLa cells. The particular promoter used can easily be determined by the person of ordinary skill in the art depending on the cell line used, based on the present disclosure.

In order to enhance the level of viral cellular products, one can also add enhancer sequences to the vector to get enhancement of the SIV LTR and/or promoter. Particular enhancer sequences can readily be determined by a person of ordinary skill in the art depending on the host cell line.

By using a series of vectors that together contain the complete SIV genome, one can create cell lines that produce a virion that is identical to the SIV virion except

that the virion does not contain SIV RNA. These virions can readily be obtained from the cells. For example, the cells are cultured and the supernatant harvested. Depending on the desired use, the supernatant containing the virions can be used or these virions can be separated from the supernatant by standard techniques such as gradient centrifugation, filtering etc.

These attenuated virions are extremely useful in preparing a vaccine. The virions can be used to generate an antibody response to SIV virions and, because these virions are identical to the actual SIV virions except that the interior of these virions do not contain the viral RNA, the vaccine created should be particularly useful. Pseudotyped virions produced from cell lines cotransfected with SIV *gag/pol* and protease genes and containing the *env* gene from another virus may be useful in creating a vaccine against this other virus. For example, an HIV *env* vector in the cell may give rise to a viral particle with an HIV *env* capable of eliciting an antibody response to HIV but without pathogenicity because of the absence of any other HIV proteins or HIV RNA.

These virions can also be used to raise antibodies to the virion that can then be used for a variety of purposes, e.g. screening for the virion, developing target systems for the virions etc. Additionally these SIV packaging-deficient cell lines can be extremely useful as a means of introducing a desired gene, for example a heterologous gene into mammalian cells, as described below.

These virions may be used as an extremely efficient way to package desired genetic sequences and deliver them to target cells infectable by SIV. This may be done by preparing a vector containing a nucleotide segment containing a sufficient number of nucleotides corresponding to the packaging nucleotides of SIV (SIV packaging region), a predetermined gene and, flanking the packaging sequence and predetermined gene, sequences corresponding to a sufficient number of sequences from within and near the LTR for packaging, reverse transcription, integration of the vector into target cells and gene expression from the vector.

The packaging region preferably corresponds to at least the region between the primer-binding site and the major 5' splice donor, as well as the region between the 5' major splice donor and the *gag* gene. With regard to the experimental data presented below concerning the packaging of such a vector, the vector might also have the first 500 bp of the *gag* gene sequence of SIV in order to enhance packaging efficiency. For example, a sufficient number of SIV sequences to be packaged,

reverse-transcribed, integrated into and expressed in the target cells would include the U3,R and U5 sequences of the LTRs, the packaging sequences and some sequences flanking the LTRs (required for reverse transcription). Although the packaging sequences described between the primer-binding site and the 5' major splice donor and those between the major splice donor and the *gag* gene would be sufficient for packaging such a vector, it may be advantageous to include the first 500 nucleotides of the gag gene coding sequence as this appears to enhance packaging further. Mutation of the *gag* initiation codon would be acceptable to avoid translation starting from this point whilst still retaining the *cis* acting *gag* nucleotide sequence required for packaging. For example, the *gag* ATG may be changed to ATC by site-directed mutagenesis.

When this vector is used to transfect one of the SIV packaging-deficient cells, it is the nucleotide sequence from this vector that will be packaged in the virions. These SIV packaged genes may then be targeted to cells infectable by SIV. This method of transformation is expected to be much more efficient than current methods. Further, by appropriate choice of genes, the method of SIV infection may be monitored.

For example, the vector could contain a sufficient number of nucleotides corresponding to both 5' and 3' LTRs of SIV to be expressed, reverse-transcribed and integrated, a sufficient number of nucleotides corresponding to the SIV packaging sequence to be packaged. The vector would also contain a sufficient number of nucleotides of the gene which is desired to be transferred to produce a functional gene (e.g. gene segment). This gene can be any gene desired, as described below.

The infectious proviral clone has been described in publications including those by Rud. It comprises a complete proviral clone in a pBluescipt KS- backbone. Using oligonucleotide site-directed mutagenesis, a series of segments in the non-coding region has been deleted between primer binding site and the *gag* initiation codon. Deletion ΔP1 includes nucleotides 171-207, ΔP2 224-256, ΔP3 304-354 and ΔP4 320-351. All of these nucleotide numberings refer to the RNA of the virus where the first base in the R region is considered +1. The proviral clone also had a reporter gene construct substituted into the envelope region for detection purposes. The constructs were transfected into Cos-1 cells and the RNA present in the cells in the virions analysed by the highly sensitive quantitative and qualitative RNase protection assay. A reduction in packaging was seen with all of the deletions. Those causing the most

severe defect in encapsidation were ΔP3 and ΔP4, reducing packaging to approximately 20% of wild type. The deletions ΔP1 and ΔP2 reduced packaging to approximately 30% of that of wild type. Thus, the SIV leader region has a packaging signal which is distributed widely across the long section of the leader between the primer-binding site and the *gag* initiation codon. In this respect, it differs from all previously documented lentiviral packaging signals. In HIV-1, virtually the whole of the packaging specificity is found between the splice donor and the *gag* initiation codon, deletions here leading to a reduction in packaging of over 90% (Lever *et al*). In HIV-2, the major packaging signal is found almost exclusively upstream of the splice donor, between the primer-binding site and the major 5' splice donor (McCann and Lever). Confirmation is thus obtained that it is not possible to predict the exact site or size of the packaging signals in lentiviruses from prior experimentation. Thus, the SIV provirus has a complete unique packaging signal and the major effect on packaging is encompassed by a region downstream of the splice donor, making it quite different from that of its closest relative (HIV-2) in which the major packaging signal is upstream of the splice donor. The present disclosure allows someone skilled in the art to construct an efficient retroviral vector system based on SIV.

The second of the accompanying drawings shows the RNA structure of the intact region in part a, on which are marked the boundaries of 4 deletion mutants that have been created: at the most extreme 5' end, there is a deletion between the two arrows marked ΔP1; in b, the resulting structure with a bar marks the site of the deleted sequence. The same is true for c for ΔP2 which clearly deletes a structure labelled DIS with some shaded bases at the tip. This is the major packaging signal. The other 3 deletions, ΔP1, ΔP3 and ΔP4, did not cause a packaging deletion, indicating that the region of the packaging signal has been precisely identified.

Deletions in the SIV 5' untranslated region

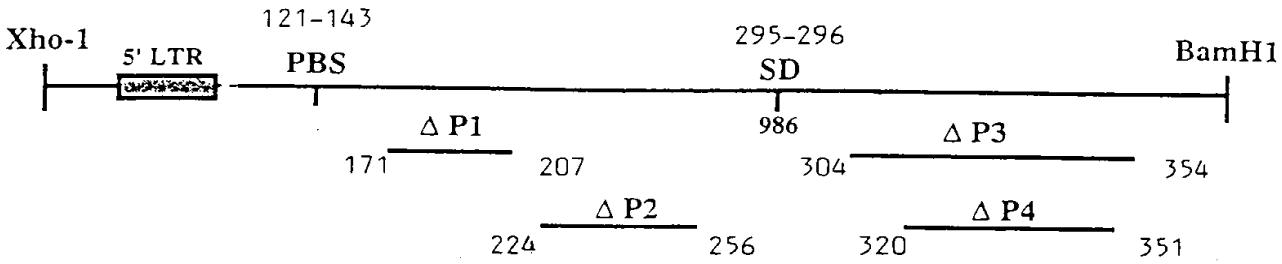
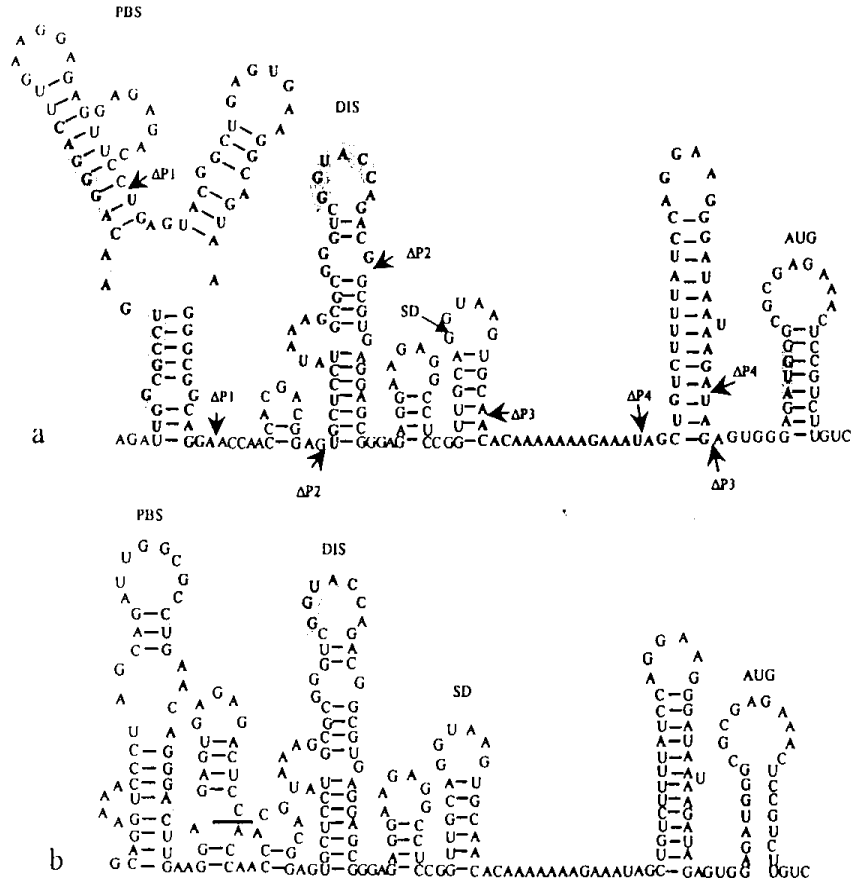


Figure 1



RNA folds showing predicted secondary structure of SIVmac leader RNA. a) wild type b) with P1 mutation c) with P2 mutation d) with P3 mutation e) with P4 mutation. Positions of deletions are shown in a) by arrows and bold type. Shaded areas indicate sequences corresponding to the primer binding site, dimerisation initiation signal and *gag* initiation codon.
Thick lines in b) c) d) and e) indicate position of the deleted sequence.

Figure 2

KT NC GBOU [illegible]

form 23/7-1 9 6.00

Agent [illegible] A Kemp [illegible]